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APOPTOSIS SIGNALING IN LEUKEMIA AND LYMPHOMA: UNDERSTANDING MECHANISMS OF CHEMORESISTANCE

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Cover: Fluorescent microscope picture of Raji cells undergoing apoptosis
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ABSTRACT

Apoptosis (programmed cell death) is a basic physiological process, essential in the balance between life and death of cells of normal tissues in the body. Apoptosis can be considered as cellular "suicide" initiated by the cell itself when infected by a virus or transformed into a cancer cell. Cancer is a genetic disease and in cancer cells the molecules involved in initiation and execution of apoptosis are frequently lost or inactivated. Blockade of apoptosis is associated with resistance to conventional cancer drugs. The importance of intact apoptosis signaling pathways in leukemia and lymphoma was addressed in the current thesis. We found that apoptotic protease activating factor 1 (Apaf-1) is required for second mitochondria activator of caspases (Smac)-dependent potentiation of protein kinase inhibitor staurosporine- and proteasome inhibitor lactacystin-triggered apoptosis in chemoresistant Burkitt lymphoma cell lines Raji and DG-75. Furthermore, the importance of elevated levels of cellular inhibitor of apoptosis 2 (cIAP2), in these cells was examined in cellular extracts from Raji cells overexpressing cytosolic Apaf-1. Subsequently cytochrome c-dependent caspase activation in Raji cells immunodepleted for cIAP2 was assessed. We found immunodepletion of cIAP2 to potentiate caspase activation only in Raji cells stably transfected with cytosolic Apaf-1. To further study the importance of Apaf-1 in response to proteasome inhibitors we used a T cell acute lymphoblastic leukemia (T-ALL) cell line, Jurkat, stably transfected with shRNA against Apaf-1. The clinically relevant proteasome inhibitor bortezomib (Velcade®) failed to induce apoptosis in Jurkat cells without Apaf-1. The bortezomib-induced apoptosis was associated with induction of pro-apoptotic factor Noxa upstream of mitochondria. Moreover, we examined primary leukemic blasts from patients with T-ALL for Apaf-1 protein expression and responsiveness to bortezomib-induced apoptosis *ex vivo*. The Apaf-1 protein expression varied amongst the different patient samples and although the sample number was low we noted the lowest increase in bortezomib-induced apoptosis in the patient sample completely deficient for Apaf-1. In order to elucidate the importance of other mediators of apoptosis, anti-apoptotic HS-associated protein X-1 (HAX-1) was assessed at the protein and transcript level in malignant lymphomas. We thus determined the mRNA expression of *HAX1* in two public transcriptomics databases. HAX-1 protein expression was assessed in a panel of 50 samples from patients with B lymphoma. We found that HAX-1 mRNA and protein was highly expressed in B lymphoma. Furthermore, we found a positive association with the proliferation marker Ki67 at the protein level in diffuse large B cell lymphoma (DLBCL) and Burkitt lymphoma samples, as well as an inverse correlation with Bcl-2 at the protein and transcript level in follicular lymphoma. Finally, the actions of the specific inhibitor of chymotrypsin-like serine proteases, TPCK and the NF-κB inhibitor Bay-11 7082 were elucidated in chemoresistant cell lines Raji and DG-75. Both compounds induced caspase-independent apoptosis as well as a decrease in constitutive NF-κB activity. Moreover, we found that TPCK and Bay-11 7082 reduced protein and mRNA expression of the NF-κB target HAX-1, which may contribute to the sensitization to apoptosis. In summary, these studies contribute to our understanding of the importance of intact apoptotic signaling pathways in sensitivity to apoptosis induced by anti-cancer substances. Studies of different pro- and anti-apoptotic molecules may lead to the identification of novel targets for therapy.

LIST OF PUBLICATIONS

I. Sun Y., Ottosson A., Pervaiz S., Fadeel B. (2007) Smac-mediated sensitization of human B-lymphoma cells to staurosporine- and lactacystin-triggered apoptosis is apoptosome-dependent. Leukemia 21:1035-1043

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IV. Ottosson Wadlund A., Jitkaew S., Johansson I., Fucharoen S., Hedenfalk I., Fadeel B. (2011) N^{α}-tosyl-L-phenylalanine chloromethylketone induces caspase-independent apoptosis in chemoresistant Burkitt lymphoma cells. *(manuscript)*

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ADDITIONAL RELEVANT PUBLICATIONS

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II. Fadeel B., Ottosson A and Pervaiz S (2008) Big wheel keeps on turning: apoptosome regulation and its role in chemoresistance. Cell Death Differ 15:443-452. [review].

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LIST OF ABBREVIATIONS

AIF	Apoptosis-inducing factor
ALL	Acute lymphoblastic leukemia
Apaf-1	Apoptotic protease activating factor-1
ATR	ATM- and Rad3-related
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma gene 2
Bcl-X_L	Bcl-2 related gene, long isoform
BH	Bcl-2 homology
Bid	BH3-interacting-domain death agonist
BL	Burkitt lymphoma
CARD	Caspase recruitment domain
Caspase	Cysteine-dependent aspartate-specific protease
CHOP	Cyclophosphamide, Hydroxydaunorubicin, Oncovin, Prednisone
Cdk	Cyclin dependent kinase
Chk	Checkpoint kinases
CED	Cell death abnormal
c-myc	cellular-myc
CNS	Central nervous system
CSC	Cancer stem cells
DEVD-AMC	Asp-glu-val-asp-7-amino-4-methyl-coumarin
DIABLO	Direct IAP-binding protein with low p*I*
DISC	Death-inducing signaling complex
DLBCL	Diffuse large B cell lymphoma
DNA	Deoxyribonucleic acid
DR	Death receptor
EBV	Epstein barr virus
EGL	Egg laying defective
Endo G	Endonuclease G
ER	Endoplasmic reticulum
FACS	Fluorescene activated cell sorter
FADD	Fas-associating protein with death domain
Fas	FS-7 associated surface antigen
FasL	Fas ligand
FDA	Food and Drug Administration
FLICE	FADD-like IL-1 converting enzyme (caspase-8)
FLIP	FLICE (caspase-8) like inhibitor protein
HAX-1	HS-(haematopoietic cell-specific)-associated protein X-1
HIV	Human immunodeficiency virus
HtrA2	High temperature requirement protein A2
IAP	Inhibitor of apoptosis protein
MALT	Mucosa-associated lymphoid tissue
MDM2	Mouse double minute 2
MEF	Mouse embryonis fibroblast
MM	Mutiple myeloma
MMP	Mitochondrial membrane potential
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NHL	Non Hodgkin Lymphoma
NOD	Nucleotide binding domain
Omi	Omi stress-regulated endoprotease
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PI	Propidium iodide
PIDD	p53-induced protein with death domain
PS	Phosphatidylserine
PUMA	P53-upregulated modulator of apoptosis
siRNA	Small/short interfering RNA
shRNA	Small/short hairpin RNA
STS	Staurosporin
tBid	Truncated Bid
TNFα	Tumor necrosis factor-α
TNFR1	Tumor necrosis factor receptor 1
TRAIL	TNF-associated apoptosis-inducing ligand
XIAP	X chromosome-linked IAP
VDAC	Voltage dependent anion channel
zVAD-fmk	Benzylcarbonyl-val-asp-val-ala-asp-flouromethyl ketone

1 INTRODUCTION

1.1 APOPTOSIS

1.1.1 Apoptosis through history and evolution

The Greek word apoptosis can be translated into the "dropping off" of petals from a flower or leaves from a tree, and was presented by Wyllie, Kerr and Currie in 1972 in an attempt to rationalize the nomenclature concerning normally occurring cell death in mammalian cells. However, the first reports on naturally dying cells in development were described already in the mid-19th century (Fadeel and Orrenius, 2005). The significant contribution of Kerr *et al* lies not only in coining the word apoptosis but they also provided a detailed morphological description of the phenomenon as well as recognized it as something not restricted to embryogenesis (Kerr et al., 1972). Since then the essential role for apoptosis not only in physiological tissue homeostasis but a central function in the immune system and in the killing of cancer cells has been extensively described. During the three following decades apoptosis or programmed cell death was established as a genetically regulated process that required ATP.

Pioneering molecular genetic studies performed in *Caenorhabditis elegans* performed largely during the 1990's provided the foundation to the identification of the main players of the apoptosis machinery in mammals. Furthermore these studies show that the cell death process is conserved throughout evolution. The discovery of the apoptosis program with cell death abnormal (*CED*)*-3*, *CED-4*, *CED-9* and Egg laying defective (*EGL)-1* genes during development in *C. elegans* (Yuan and Horvitz, 1992, Yuan et al., 1993, Hengartner and Horvitz, 1994, Conradt and Horvitz, 1998) rendered the Nobel prize in Physiology or Medicine in 2002. Studies in *Drosophila melanogaster* have further confirmed the existence of a conserved pathway of apoptosis (Meier and Vousden, 2007).

1.1.2 Intrinsic and extrinsic apoptosis signaling

Genetic and biochemical studies have identified two major pathways to programmed cell death that are largely independent; the extrinsic or the receptor-mediated pathway and the intrinsic or mitochondria-dependent pathway (Figure 1). The extrinsic pathway is activated upon binding of the designated ligand to a death receptor. This induces the recruitment of the adaptor molecule, a receptor-associated death domain and pro-caspase-8/10, which results in the formation of the death-inducing signaling complex (DISC). DISC formation leads to the processing and activation of pro-caspase-8/10 and the initiation of a cascade of proteolytic activity within the cell. Cells in which caspase-8 is strongly activated at the DISC, leading to the direct activation of caspase-3 and subsequent cell death, are classified as type I cells (Scaffidi et al., 1998). However, cells can also undergo apoptosis with reduced death receptor stimulation and no direct activation of pro-caspase-3 by caspase-8. Such cells are classified as type II cells, and require mitochondrial amplification of the initial death stimulus (Scaffidi et al., 1998). Thus, in the intrinsic or mitochondria-dependent apoptosis pathway, caspase-8

mediates the cleavage of Bid (a member of the Bcl-2 family) which is translocated to mitochondria, leading to a conformational change and oligomerization of the mitochondria-associated Bak and the cytosolic Bax forming pores in the outer mitochondrial membrane (Hsu et al., 1997, Griffiths et al., 1999). From the mitochondrial intermembrane space pro-apoptotic molecules such as the apoptosis inducing factor (AIF), second mitochondrial activator of caspases (Smac/DIABLO), Omi/HtrA2, Endonuclease G (Endo G) and cytochrome *c* are released (Danial and Korsmeyer, 2004). Subsequently, cytochrome *c* induces an ATP driven conformational change of apoptotic protease-activating factor-1 (Apaf-1) forming a heptamer apoptosome complex (Fadeel et al., 2008). Within the apoptosome caspase-9 is activated by dimerization (Zou et al., 1999). Caspase-9, in turn, activates downstream effector caspases such as procaspase-3 and -7, resulting in apoptosis. Activation of caspase-3, -7 and -9 can be inhibited by members of the inhibitors of apoptosis proteins (IAP) family. In turn the IAPs are negatively regulated by binding of Smac (Figure 1). The intrinsic pathway is not only activated by cleavage of Bid but also directly by apoptotic stimuli such as cytotoxic compounds, DNA damage, growth factor deprivation etc. (Danial and Korsmeyer, 2004), summarized as cellular "stress" in Figure 1.

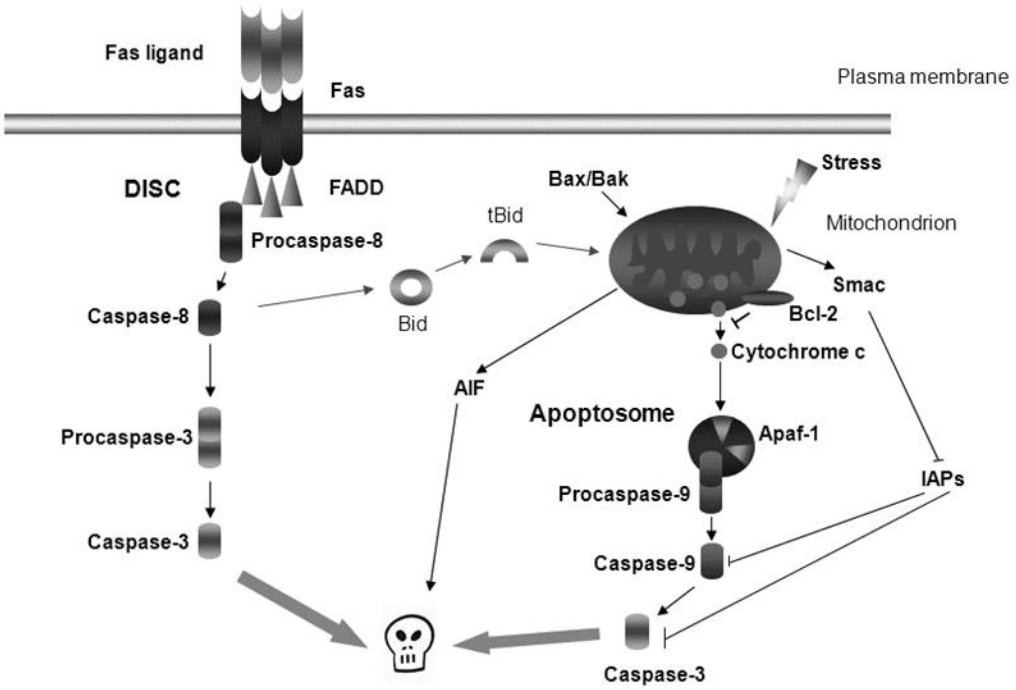

Figure 1. Extrinsic and intrinsic apoptosis signaling. Consult text for details. DISC, death inducing signaling complex

1.1.3 Death receptors

Tumor necrosis factor receptor 1 (TNFR1), Fas, TNF-related apoptosis-inducing-ligand (TRAIL) receptor are all cysteine-rich cell surface receptors related to the low-affinity nerve growth factor family. The extracellular region of these TNF receptor family members carries two to six repeats of cysteine-rich motifs. These receptors also share homology in their cytoplasmic domain which, in turn, is sufficient to trigger apoptosis when overexpressed alone, has been termed the death domain (Fulda and Debatin, 2004). The TNFR1 also known as Death Receptor 1 (DR1) is involved in a number of physiological processes such as cytotoxicity, hepatocyte proliferation and in mediating inflammatory responses. Its designated ligand TNF efficiently activates TNR1-dependent signaling activating downstream signaling cascades, e.g., activating NF-κB and c-Jun N-terminal kinase (JNK) (Schwenzer et al., 1999). Death receptor 2 (also termed CD95/APO-1/Fas) is the most studied and best characterized death receptor. Fas was identified in 1989 as a cell surface molecule involved in apoptosis (Trauth et al., 1989) and its ligand CD95L/FasL a few years later (Takahashi et al., 1994). Fas is highly expressed in activated lymphocytes and in a variety of lymphoid and non-lymphoid tissues. FasL plays an important role in regulation of the immune system by triggering autocrine or paracrine cell death by activated T lymphocytes (Krammer, 2000). Upon FasL binding to Fas, the receptor oligomerizes and the death domain-containing adaptor molecule FADD is recruited together with pro-caspase-8, pro-caspase-10, and the cellular FLICE inhibitory protein (FLIP) (Kischkel et al., 1995). DR4 and DR5 represent the tumor-related apoptosis inducing ligand receptor 1 and 2, respectively. The ligand, TRAIL, is a type-II transmembrane protein showing high homology to FasL (Wiley et al., 1995) and induces caspase-dependent apoptosis upon binding to the receptor in a similar manner to Fas/FasL (Krammer, 2000). Death ligands to all of the above mentioned death receptors have been demonstrated to interact with decoy receptors that lack the death domain and thus cannot form signaling complexes (Mahmood and Shukla, 2010).

The role of death receptors and their ligands in cancer and cancer treatment has been extensively studied. When interrogating the *in silico* transcriptomics (IST) data base (Kilpinen et al., 2008) for the mRNA expression levels of Fas and FasL, elevated expression level is associated to hematological malignancies such as lymphoma and chronic lymphoblastic leukemia (Ottosson Wadlund, unpublished observation). In contrast, solid tumors such as breast cancer and head and neck cancer display increased transcript levels of TRAIL. Information on the expression of TRAIL receptor DR4 (*TNFRSF10A*) is currently unavailable due to lack of probes on the assessed chip. However, relative mRNA expression of DR5 (*TNFRSF10B)* can be retrieved and is elevated in chronic lymphoblastic leukemia, mesothelioma, adrenal gland cancer and bladder cancer (Ottosson Wadlund, unpublished observation).

1.1.4 Caspases

At the center of the apoptotic machinery of mammalian cells a family of cystein proteases can be found. The name cystein-dependent aspartate-specific proteases

implies their enzymatic specificity, dominant for protein substrates containing Asp with the use of a cysteine side chain for catalyzing peptide bond cleavage. Members of the caspase family are conserved throughout evolution and the pro-apoptotic *CED-3* gene was first described in *C.elegans* where it is absolutely necessary for apoptotic suicide. Caspases are essential for the maintenance of tissue homeostasis during development and especially during development of the central nervous system (CNS) and the heart. Knockout mice of caspase-3 and -9 die prenatally to a high extent and demonstrate severe enlargement and malformation of the brain (Kuida et al., 1996, Kuida et al., 1998). Furthermore, isolated primary thymocytes from these knockout mice displays decreased sensitivity towards apoptotic stimuli (Kuida et al., 1996, Kuida et al., 1998, Woo et al., 1998). Targeted disruption of the *CASP8* gene in mice produced a phenotype of *in utero* lethality and impaired heart muscle development (Varfolomeev et al., 1998). In ovaries of caspase-2 knockout mice an excess number germ cells were found, and caspase-2-deficient oocytes exhibited almost complete resistance to apoptosis. Moreover, B-cells from caspase-2-deficient mice were insensitive to granzyme B-induced apoptosis (Bergeron et al., 1998). Taken together, the importance of different caspases during development seems to be tissue specific, and the apoptotic response to different stimuli also reflects cell type specificity.

To date, all 15 mammalian caspases described are related to the pro-inflammatory interleukin-1β converting enzyme (caspase-1) involved in cytokine processing. Caspases are thus involved in different biological processes, primarily inflammation (e.g., caspase-1, -4 and -5) and apoptosis where they are sub grouped as initiator/apical (e.g., caspase-2, -8, -9 and -10) or as executioner/effector (e.g., caspase-3, -6, and -7), but also keratinocyte differentiation (e.g., caspase-14) and suggested roles in proliferation and migration (Thornberry et al., 1992, Nicholson and Thornberry, 1997). Recently the 15th member of the family of caspases was identified in pig, dog, and cattle, demonstrating pro-apoptotic activity (Eckhart et al., 2005).

Caspases are abundantly expressed as inactive proforms or zymogens with regulatory N-terminal prodomains in the mammalian cell. All caspases comprise a regulatory N-terminal domain as well as a catalytic domain with a large and a small subunit (Nicholson and Thornberry, 1997). Initially it was thought that all caspases where activated by proteolysis; however, recently is has become more and more evident that this is applicable first and foremost to the executioner caspases (caspase-3, -6, and -7). Initiator caspases (caspase-8, -9 and -10) require dimerization facilitated by recruitment to oligomeric activation platforms formed in response to apoptotic stimuli. This recruitment leads to a local increase in caspase concentration which in turn results in activity by proximity-induced dimerization (Muzio et al., 1998, Salvesen and Dixit, 1999). The initiator caspases are specific to different activation platforms; the Death DISC activates caspase-8 and -10 via the caspase recruitment domain (CARD); the apoptosome activates caspase-9 via the death effector domain (DED); and the PIDDosome (PIDD, p53-induced protein with death domain) activates caspase-2 via the CARD (Chang et al., 2003). During the past decade increasing information on the dimerization and activation of inflammatory caspases has been reported. The

oligomeric platforms with affinity for the CARD prodomains are called inflammasomes and when dysregulated have been associated with inflammatory diseases such as Gout and Type 2 Diabetes (Schroder and Tschopp, 2010). Executioner (or short prodomain) caspases occur as inactive dimers that are activated upon cleavage of the catalytic domain (Salvesen and Dixit, 1999).

Although the amino acid sequence of caspase substrates differs they are among the most specific of selective proteases and cleave their target protein after Asp residues. To date, all caspases described share one preferred cleavage site at the C-terminal of a four amino acid motif, X-X-X-D (X representing any amino acid and D representing Aspartic acid). Furthermore, among the different subsets of caspases high protein identity is found, resulting in similar substrate preferences. Inflammation-oriented caspases (caspase-1, -4, and -5) prefer the tetrapeptide sequence WEHD, initiator caspases (caspase-8, -9 and -10 with the exception of caspase-2) prefer the tetrapeptide sequence (I/L/V)EXD, and executioner caspases (caspase-3, -6, -7 and -2) prefer the tetrapeptide sequence DEXD (Denault and Salvesen, 2002).

The activation of the proteolytic cascade of caspases is irreversible and thus requires strict regulation within the cell. Endogenous inhibitors of caspases are members of the Inhibitors of Apoptotic Proteases (IAP) family and will be discussed below. In addition to endogenous regulators the virally encoded cowpox virus CrmA and baculovirus p35 proteins are able to suppress caspase-mediated host-responses (Denault and Salvesen, 2002).

Functional consequences of mutations and deletions of initiator and executioner caspase genes in tumorigenesis have been widely discussed in the literature. In a study by Teitz and colleagues it was demonstrated that low protein expression of caspase-8 was the result of gene silencing of the *CASP8* gene due to promoter methylation in childhood neuroblastoma (Teitz et al., 2000). Moreover, other mutations of *CASP8* and *CASP7* have been found in colorectal cancer as well as in head and neck cancer. Furthermore, decreased expression of caspase-3 on protein and transcript level has been associated to chemoresistance in breast adenocarcinoma (Olsson and Zhivotovsky, 2011). Mutations or deletions of single caspases may not play a significant role in the development of a tumor cell phenotype. However, the central role of caspases in tumorigenesis may lie in the joint contribution of expression pattern changes of different caspases (Olsson and Zhivotovsky, 2011).

1.1.5 Inhibitors of apoptosis (IAPs)

The prototype IAP was first described in 1993 in a genetic screen of the baculoviral genome to identify regulators of host-cell viability during virus infection (Crook et al., 1993). The human IAP family has since then grown and are today considered to consist of eight members, where the X-linked Inhibitor of Apoptotic Proteins (XIAP), is the best characterized member. The IAP family also includes cellular inhibitor of apoptosis

proteins 1 (c-IAP1), cellular inhibitor of apoptosis proteins 2 (c-IAP2) (Duckett et al., 1996, Uren et al., 1996) and Survivin among others (Ambrosini et al., 1997). The IAPs contain one-three Baculovirus IAP Repeat (BIR), which signifies the IAP family, and an E3 ligase activity domain, the RING domain. The regions closely related to the BIR2 of XIAP specifically targets caspases-3 and -7, and regions closely related to the BIR3 specifically targets caspase-9. Moreover, the E3 ligase activity of the RING domain results in poly-ubiquitination of proteins targeting them for proteasomal degradation. However, ubiquitination does not always result in targeted degradation: in some situations, ubiquitination can modify the biological activity or subcellular localization of a protein. Consequently, the primary target of RING domain-mediated ubiquitination in XIAP is direct polyubiquitination of a lysine(s) outside the RING domain of XIAP (Yang et al., 2000). Furthermore, members of the IAP family are strictly regulated on a transcriptional level. For example, c-IAP2 and XIAP are regulated by the pro-survival transcription factor nuclear factor of κ B (NF-κB).

IAPs can also be regulated by pro-apoptotic factors and biochemical studies have led to the identification of the intermembrane space protein, second mitochondrial activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO). On its release from mitochondria mature Smac/DIABLO binds XIAP, displacing caspases from XIAP. Smac/DIABLO can bind two distinct regions of XIAP. Residues 56–59 of Smac/DIABLO are homologous to the exposed amino-terminal motif that is used by caspase-9 to bind BIR3 of XIAP, and Smac/DIABLO has been found to bind to the same pocket in XIAP, thereby displacing caspase-9 from the complex (Srinivasula et al., 2001).

XIAP protein has been shown to be highly expressed in Reed-Sternberg cells of Hodgkin lymphoma samples (Kashkar et al., 2003). Moreover, using the IST data base (Kilpinen et al., 2008) the relative mRNA expression of *BIRC4* (XIAP) is increased primarily in hematological malignancies such as acute and chronic leukemia and myeloma. Moreover, solid tumors such as prostate and bladder cancer displays elevated transcript levels of *BIRC4* (Ottosson Wadlund, unpublished observation).

1.1.6 Apaf-1 and the apoptosome

The (apoptotic protease activating factor-1) *APAF1* gene is a human CED-4 homologue giving rise to at least three distinct splice forms (Shi, 2001). The *APAF1* gene encodes a cytoplasmic protein containing several copies (12-13) of the WD-40 domain at the C-terminus, a CED-4 homology ATPase domain (CED-4 like or NB-ARC) and a CARD at the N-terminus (Zou et al., 1997) (Figure 2). The protein was identified using cytosolic fractionation of HeLa cells in search of activators of caspase-3 and belongs to a family of nucleotide-binding and oligomerization domain (NOD) containing proteins (Zou et al., 1997). Most mammalian NOD proteins are involved in innate immune response signaling, Apaf-1 excluded, but they all oligomerize to execute their function as signal transducers (Schroder and Tschopp, 2010). Mice deficient for Apaf-1 die perinatal and the embryos displays morphological abnormalities of the brain and the

craniofacial compartment, similar to the ones of caspase-9 knockout mice. Moreover, primary embryonic fibroblasts from the Apaf-1 knockout mice examined for caspase-2, -3, -8 activity showed decreased activation in response to a variety of apoptotic stimuli (Cecconi et al., 1998, Yoshida et al., 1998). These studies show the fundamental role of the Apaf-1-dependent apoptosis pathway during CNS development.

Cytochrome *c*, part of the respiratory chain and crucial for energy production in mitochondria, was identified as an important cofactor for the activation of caspase-3 by Soengas and colleagues and the cellular receptor for cytochrome c was later identified as Apaf-1 (Soengas et al., 2001). Cytochrome *c* and dATP were found to be essential cofactors for the formation of a ~ 700 kDa Apaf-1/caspase-9 complex, the apoptosome (Chou et al., 1998). Upon binding of cytochrome *c* Apaf-1 undergoes conformational changes promoting oligomerization and delocalization of the CARD domain. Oligomerization of Apaf-1 monomers arranges the CARD domains centrally in a wheel shaped structure (Figure 2). Crystal structure of murine Apaf-1 revealed how the monomer is kept in its autoinhibited conformation with ADP relying on an Arg residue in close proximity to the bound nucleotide (Reubold et al., 2011). The central hub of CARD domains provides a platform for procaspase-9 zymogen binding via the CARD domain of procaspase-9, resulting in subsequent activation of procaspase-9 (Chou et al., 1998, Qin et al., 1999). Subsequently downstream effector caspases, such as caspase-3 and -7 are activated resulting in cell death.

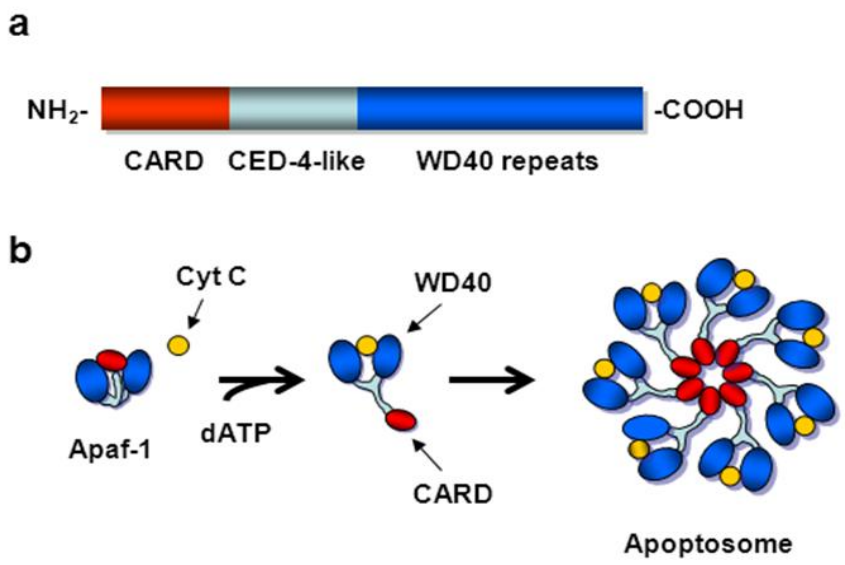

Figure 2. (a) The Apaf-1 protein structure. (b) Apoptosome formation. Adapted from (Fadeel et al., 2008).

Apoptosis signaling downstream of mitochondria is regulated on multiple levels and the apoptosome is no exception. Positive and negative regulation of the apotosome by endogenous as well as exogenous factors was recently reviewed by Fadeel and co-workers (Fadeel et al., 2008). Proteins directly interacting with Apaf-1 and negatively regulates apoptosome dependent signaling includes heat shock protein 70 and 90, while Nucling positively regulates apoptosome dependent signaling as reviewed by (Schafer and Kornbluth, 2006). Furthermore, mislocalization of the Apaf-1 protein, from the cytosol to other cellular compartments has been reported to negatively regulate apoptosome-dependent apoptosis in chemoresistant cell lines (Besse et al., 2004, Sun et al., 2005).

As mentioned above Apaf-1 is subjected to transcriptional modification by differential splicing (Benedict et al., 2000). Epigenetic modification by hypermethylation of the *APAF1* gene was reported in neuroblastoma recently (Grau et al., 2011) and hypermethylation of the *APAF1* promoter has been observed in a variety of different types of cancer, including acute leukemia (Fadeel et al., 2008). The consequence of these epigenetic changes was demonstrated to be associated to chemoresistance and survival. Moverover, inhibition of histone deacetylase activity resulting in increased binding of E2F-1 and p53 to the *APAF1* promoter increased the Apaf-1 protein expression and induced apoptosis in retinal cells has been reported (Wallace and Cotter, 2009).

1.1.7 The Bcl-2 protein family

The *BCL2* gene was originally discovered as an oncogenic gene at the chromosomal breakpoint in B-cell lymphoma carrying a t(14;18) chromosomal translocation (Tsujimoto et al., 1984). Subsequent studies found that Bcl-2 had potent anti-apoptotic activity and was capable of inhibiting apoptotic cell death induced by many different death signals (Adams and Cory, 1998). The *BCL2* gene is essential for lymphoid development and a large number of proteins have been identified that belong to the *BCL2* gene family. Bcl-2-related proteins, the BH3-only proteins, are recognized as key regulators of apoptosis and present both pro- and anti-apoptotic features. The anti-apoptotic members of the Bcl-2 family (Bcl-1, Bcl-x_L, Bcl-w, Mcl-1, A1, Boo/Diva/Bcl-2L10, Bcl-B and *C.elegans CED-9*) all contain three or four characteristic BH regions of homology (Bcl-2 Homology domains). Pro-apoptotic members of the Bcl-2 family are sub grouped according to their biochemical function and to their protein structure: containing two-three BH domains (Bax, Bak, Bcl-x_S, Bok and Bcl-G_L) or only one short BH3 domain (Bad, Bik, Blk, Bid, Hrk/DP5, Bim, Bmf, Noxa, Puma and *C.elegans EGL*-1) (Chonghaile and Letai, 2008).

To avoid undesired activity the BH3-only proteins are tightly regulated, both transcriptionally and post-translationally. In *C.elegans* the *EGL-1* gene is under transcriptional control by the repressor TRA-1A (Conradt and Horvitz, 1999). In mammals Noxa and Puma can both be regulated by p53 (Oda et al., 2000, Han et al.,

2001), and the JNK-pathway has been shown to transcriptionally upregulate Bim in neurons (Harris and Johnson, 2001). Bim also undergo conformational changes upon apoptotic stimuli, releasing it from the dynein light chain of the microtubule and allowing it to translocate to mitochondria. (Puthalakath et al., 1999). Another example of post-translational modification of BH3-only proteins is the myristoylation of the truncated p15 tBid after cleavage by caspase-8, promoting its translocation to the mitochondria (Zha et al., 2000). Phosphorylation of Bad renders it inactive in a complex with 14-3-3ι scaffold proteins, preventing it to bind to pro-apoptotic Bcl-2 family members (Zha et al., 1996).

Transcript levels of *BCL2* are elevated in human hematological malignancies according to the IST data base (Kilpinen et al., 2008). Moreover, protein expression of Bcl-2 has been demonstrated to be high in hematological malignancies (e.g., follicular lymphoma) (Reed, 2008). Several mechanisms have been suggested to account for this, including the chromosomal translocation t(14; 18) in patients with follicular lymphoma (Tsujimoto et al., 1984). Nonetheless, Bcl-2 overexpression has also been linked to gene amplification, hypermethylation of the *BCL2* gene and decreased levels of pro-apoptotic BH3-only proteins (Chonghaile and Letai, 2008).

1.1.8 Caspase-independent cell death

As described above apoptosis is normally orchestrated by the activation of caspases and subsequent chromatin condensation and DNA fragmentation. Nevertheless, cells have been shown to die without caspase activation and coincided with the release of mitochondrial factors such as AIF, Omi and Endo G (Kroemer and Martin, 2005). Upon treatment with substances like staurosporine AIF is cleaved from its membrane anchoring in the mitochondria. The 57 kDa AIF fragment can then be released to the cytosol upon mitochondrial membrane permeabilization. AIF subsequently translocates to the nucleus where it is involved in large-scale DNA fragmentation and cell death (Joza et al., 2001). Endo G is another mitochondrial factor that is released during cell death and translocates to the nucleus. It has been demonstrated to participate in DNA degradation in *C.elegans* (Parrish and Xue, 2003). Furthermore, the mitochondrial serine protease Omi/HtrA2 can mediate both caspase-dependent and caspase-independent apoptosis by cleavage of IAPs, the anti-apoptotic protein HAX-1 and Ped/Pea-15 (an inhibitor of the DISC and of stress kinase (Kroemer and Martin, 2005).

1.1.9 HS-1-associated protein X-1 (HAX-1)

By using a yeast two-hybrid screen with the multifunctional hematopoietic cell-specific protein 1 (HS1) Suzuki and colleagues identified the HS1-associated protein 1 (HAX-1) (Suzuki et al., 1997). HAX-1 is a 35-kDa protein that is associated with the cytosolic side of mitochondrial and endoplasmic reticulum (ER) membranes, but lacks a proper transmembrane domain (Jeyaraju et al., 2009). The *HAX1* gene regulation is still elusive. However, the expression of HAX-1 has been shown to be induced by cytokines (Chao et al., 2008) and recently it was demonstrated that HAX-1 is under transcriptional control of NF-κB (Jitkaew et al., 2009). Moreover, HAX-1 can be

subjected to post-transcriptional modifications and to date, eight human splice variants (Lees et al., 2008) and seven rat splice variants have been identified (Grzybowska et al., 2006).

HAX-1 has been shown to interact with a number of cellular and viral proteins as well as mRNA, and be involved in multiple signaling pathways and cellular processes. The overall expression of HAX-1 has displayed relatively high abundance among different tissues and this might reflect the miscellaneous roles of HAX-1 (Fadeel and Grzybowska, 2009). Numerous studies have implicated the role of HAX-1 in apoptosis and its functional association with apoptosis-regulating proteins and organelles. A clinically relevant example of the anti-apoptotic functions has been demonstrated in severe congenital neutropenia (Kostmann disease), where homozygous *HAX1* mutations were identified (Klein et al., 2007). Specific mutations of *HAX1* were shown to be associated with neurological and neuropsychological abnormalities in Kostmann patients (Carlsson et al., 2008). Furthermore, HAX-1 have been implicated in B cell receptor (BCR)-mediated apoptosis, and in blocking of Bax-induced apoptosis as well as in inhibition of caspase-9 mediated apoptosis (Fadeel and Grzybowska, 2009). In addition, it was recently reported that the mitochondrial serine protease Omi/HtrA2 was able to activate autophagy by down regulation of the protein expression of HAX-1, which in turn regulates Beclin-1 (Li et al., 2010). Another important physiological function where the role of HAX-1 has been suggested is in the regulation of cell migration. HAX-1 has been demonstrated to interact with F-actin associated protein cortactin in a polycystic kidney disease model (Gallagher et al., 2000). Moreover, HAX-1 was able to bind the β6 subunit of V_6 intergrins and thereby regulate clathrin-mediated endocytosis in an oral squamous cell carcinoma model (Ramsay et al., 2007). HAX-1 also regulates neutrophil adhesion and motility (Cavnar et al., 2011). These studies, might suggest that HAX-1 is important for metastatic tumor progression.

1.1.10 Additional apoptotic regulators

Calpains are a family of calcium-dependent cysteine proteases consisting of the μ-calpain or calpain-1 and the m-calpain or calpain-2 expressed in all tissues as well as number of tissue specific calpains. Calpain activity has been shown to be involved in a number of physiological processes such as cytoskeletal remodeling, cellular signaling, apoptosis and cell survival. The endogenous inhibitor calpastatin provides negative regulation in a calpain-calpastatin complex (Ono et al., 1998). The most common cellular compartment is the cytosol although calpains have recently been demonstrated to exist in mitochondria where it can cleave mitochondrial aspartate aminotransferase and AIF (Ozaki et al., 2007, Norberg et al., 2008). Mitochondrial μ-calpain has been suggested to be an initiator of AIF-induced cell death and m-calpain has been proposed to be involved in the release of truncated AIF to the cytosol (Ozaki et al., 2008, Ozaki et al., 2009).

Granzymes are family of serine proteases released by cytotoxic members of the immune response, e.g., cytotoxic T cells (CTL) and NK cells in clearance of infected or

transformed cells. The cytotoxic granules of CTLs and NK cells contain a number of different granzymes as well as the pore-forming protein perforin. However, amongst granzymes the primary apoptosis mediator is granzyme B (GrB). Once in the target cell GrB is able to trigger several apoptotic pathways by proteolytic cleavage of death substrates. GrB has been demonstrated to activate both initiator and executioner caspases (Medema et al., 1997, Darmon et al., 1995) and to mediate direct cleavage of Bid and disruption of the Mcl-1/Bim complex (Heibein et al., 2000, Han et al., 2005).

Lysosomes are organelles involved in degradation of cellular macromolecules and lysosomal hydrolases were long thought to induce only necrotic cell death. However, in 1998 rat myocytes were shown to undergo cathepsin D-induced apoptosis in response to reactive oxygen species (Roberg and Öllinger, 1998). Cathepsin D is released after lysosomal membrane permeabilization triggered by a large number of different apoptotic stimuli such as reactive oxygen species. In addition, cathepsin D has been shown to be involved in TNFα-mediated apoptosis as well as in direct cleavage of Bid (Repnik et al., 2011).

1.2 HEMATOLOGICAL MALIGNANCIES

Even though the entity of leukemic disease has been known since the mid 1800 the causes are still not completely uncovered. Epidemiological studies have demonstrated that ionizing irradiation, chemical carcinogens, certain viral infections and genetic predisposition are important but not essential factors in the development the DNA changes prerequisite for different types of leukemia/lymphoma. Nevertheless, leukemic disease can be defined as a clonal expansion of a certain leukocyte at a specific state of differentiation, with an intact ability to proliferate and circumvent cell death (Lerner, 2011). The characteristic DNA damages that results in the malignant features of hematological neoplasms are mainly the result of one or more changes in genes controlling functions such as the cell cycle (e.g., *TP53*, *RB1* etc.), apoptosis (e.g., *BCL2*, *MYC* etc.), signal transduction (e.g., *BCR/ABL*, *RASA1* etc.) (Mitelman et al., 2007). The malignant phenotype can also be a result of viral infections by such as the Epstein Barr virus (EBV) and the human T-lymphotropic virus type I (HTLV-I). All hematological neoplasms are characterized by a blockage at a certain state of differentiation. In general acute leukemias and high grade proliferating lymphomas can be classified as neoplasms with changes in processes controlling proliferation, while chronic lymphoblastic leukemias and low grade non-Hodgkin lymphomas can be classified as neoplasms with changes in processes controlling survival and/or cell death (Lerner, 2011). Molecular genetic analyses of single proto oncogenes and tumor suppressor genes in hematological malignancies, have demonstrated that translocations occurs at an early state of the disease. On the other hand, point mutations and deletions of tumor suppressor genes such as *TP53* or point mutations in oncogenes such as *RASA1* occur at a much later state of the disease (Britschgi and Fey, 2009).

1.2.1 The p53 family

In 1979 a 53 kDa protein discovered was initially thought be an oncogene (Lane and Crawford, 1979), but has since then been reevaluated as one of the most important human tumor suppressor genes. The p53 protein family is a family of transcription factors including p53 and its homologues p63 and p73 (Graziano and De Laurenzi, 2011). The *TP53*, *TP63* and *TP73* genes all encode several protein isoforms due to alternative promoters and differential splicing, which results in functional overlap to a certain extent. p63 has been demonstrated to be essential for maintenance and survival of epithelial stem cells (Graziano and De Laurenzi, 2011), while p73 is essential for survival and migration of neurons (Pozniak et al., 2000).

The p53 knock-out mouse does not only demonstrate a high rate of spontaneous tumors but also display defects in the neural tube resulting in midbrain exencephaly and *in utero* death of the fetus (Donehower et al., 1992, Armstrong et al., 1995). Moreover, the *TP53* gene is mutated in about 50% of all human tumors, and in about 10-15% of all leukemias (Mitani et al., 2007). *TP53* status is associated with sensitivity towards anticancer drugs and radiotherapy (Lane, 2005). Upon cellular stress including DNA damage and oncogene activation p53 accumulates and triggers transcriptional activation of p53 target genes such as p21, GADD45, Bax, Puma, Noxa, which in turn leads to cell cycle arrest, senescence and apoptosis (Landesman et al., 1997, McCurrach et al., 1997, Nakano and Vousden, 2001). Moreover, p53 has been shown to induce apoptosis of cancer cells by activation of caspase-2 via transcriptional upregulation of PIDD (Tinel and Tschopp, 2004). Furthermore p53 induces transcription of the MDM2 gene, producing a protein that ubiquitinates p53, targeting it for proteasomal degradation (Lai et al., 2001).

1.2.2 Malignant lymphomas

Lymphomas are a group of malignant tumors typically presented as a solid tumor of lymphoid cells that most commonly originates from lymph nodes. Lymphoma can also involve other organs, e.g., the skin, brain bowl and bone, referred to as extranodal lymphoma. Every year 2000 new cases are presented in Sweden, and lymphoma is one of the ten most commonly occurring cancers (Bergman et al., 2010). There are about 20 subgroups of lymphomas, and the six most common types are Diffuse Large B cell lymphomas (DLBCL), Chronic Lymphoblastic Leukemia (CLL), Follicular Lymphoma, Hodgkin Lymphoma, Immunocytoma, Mantle Cell Lymphoma are all of B-cell origin. Less common are for example Burkitt Lymphoma, Anaplastic Lymphoma, and Mucosa Associated Lymphoid Tissue Lymphoma (MALT) (Bergman et al., 2010). Survival of lymphoma patients is associated to the anatomical spread of the tumor which is classified by the Ann Arbor classification (Carbone et al., 1971).

1.2.3 Burkitt lymphoma

Burkitt lymphoma (BL) was first characterized as a lymphosarcoma by Irish surgeon Burkitt in 1958 (Burkitt, 1958). It can be described as a highly aggressive form of non-Hodgkin B cell lymphoma most commonly occurring in young adults or children. BL

accounts for 40-50% of all childhood non-Hodgkin lymphoma. There are several different variants of BL including endemic, sporadic and immunodeficiency associated. Nevertheless, they all share the common characteristic hallmark of chromosomal translocation of the *MYC* oncogene (Yustein and Dang, 2007). BL is a malignancy of intermediate size B cells that infiltrate nodal or extranodal tissue in a diffuse pattern but depending on the variant can be presented differently. In endemic areas (i.e., equatorial Africa) there is a high tendency for the involvement of the facial bones in young children while in other geographic areas most patients present with abdominal tumors. Moreover the endemic variant is associated EBV and concomitant malaria infection (Wright, 1999).

An unconditional feature of BL is the *MYC* translocation which, most commonly (approximately 85%) is the result of translocation of *MYC* from chromosome 8 to the IgH enhancer elements on chromosome 14 (t(8;14)) driving an inappropriate expression of c-myc. Although several translocations have been described in BL, including t(2;8) and t(8;22), they all result in deregulated expression of c-myc (Pelicci et al., 1986). At the same time the t(8;14) translocation may also be found in DLBCL and over the past decade the World Health Organization (WHO) has continuously updated the criteria for distinguishing BL from Burkitt-like lymphomas and DLBCL. With the introduction of transcriptional and genomic profiling using microarray techniques the molecular diagnosis of BL has improved and can be coupled to clinical outcome (Hummel et al., 2006). Initially BL was associated with poor outcome, but since the introduction of high intensity chemotherapy, the outcome has improved significantly. Currently, the initial therapeutic regime normally used is CHOP consisting of cyclophosphamide, vincristine, doxorubicin and prednisolone, but this can also be combined with high-dose methotrexate and etoposide etc. The CHOP regime corresponds to a typical therapeutic strategy in childhood malignancies and is used in treatment of both children and adults (Magrath et al., 1996). Recently the use of monoclonal antibodies as adjuvant in treatment of BL has become more frequent and resulted in favorable clinical outcomes. Among these anti-CD20 (i.e., rituximab) has been demonstrated to induce cytotoxicity by antibody-dependent as well as cell-dependent mechanisms (Meinhardt et al., 2010).

1.2.4 Myc

The *MYC* proto-oncogene family (comprising c-myc, N-myc, and L-myc*)* ranks among the most studied group of genes in the literature. This family comprises of transcription factors of the basic region/helix-loop-helix zipper (bHLHZip) family that can heterodimerize with Max, another bHLHZip protein. It is believed that Myc exerts its main functions through gene regulation by recruiting transcriptional cofactors involved in modulation of RNA polymerase II function and of chromatin structure, including histone acetyl transferase complexes (Grandori et al., 2000). In normal cells, cellular processes including proliferation, growth, apoptosis, energy metabolism, and differentiation are under modulation by Myc proteins in response to external signals. The c-myc expression is induced by multiple mitogenic signaling pathways including

Wnt, Notch, STAT, receptor tyrosine kinases (RTKs), as well as hormone receptor pathways (Eilers and Eisenman, 2008). The Myc proteins are turned over at a very high rate via the ubiquitin/proteasome pathway involving at least three types of E3 ligases; SCF^{Fbw7}, SCF^{Skp2} and HectH9 (von der Lehr et al., 2003, Adhikary et al., 2005).

In response to extrinsic and intrinsic signals it has been suggested that Myc regulates 10-15% of all genes and among its targets cell cycle regulatory genes, including cyclins, cyclin-dependent kinases (Cdk), Cdk inhibitors (CKI), E3 ubiquitin ligase components targeting CKIs and replication proteins can be found (Grandori et al., 2000). Myc has also been demonstrated to regulate sensitivity towards apoptosis. In response to a variety of different stimuli such as ligation of death receptors, serum deprivation, hypoxia and cytotoxic drugs Myc has been shown to induce apoptosis (Eilers and Eisenman, 2008). Although the exact mechanisms by which Myc induces apoptosis remains to be elucidated, Myc induces the expression of the tumor suppressor protein p14Arf, which activates p53 by binding and sequestering the Mdm2 E3 ligase (Eischen et al., 1999). p53 in turn activates pro-apoptotic genes including *BAX* and *BBC3* (Puma) as well as mediators of cell cycle arrest (e.g., *p21CIP1*).

Activated *MYC* oncogenes contribute to the development of a wide variety of human cancer including leukemia, lymphoma and solid tumors, as a consequence of translocation, amplification point mutations or defects in upstream regulators. However deregulation of Myc also triggers intrinsic tumor suppressor mechanisms including activation of p53. The importance of apoptosis in Myc-induced tumor development has been well established. Thus, loss of pro-apoptotic genes including *TP53*, *ARF*, *BAX* and *BIM* or overexpression of anti-apoptotic genes such as *BCL2L1* and *BCL2* cooperates with *MYC* and accelerate tumorgenesis (Strasser et al., 1990). In contrast, the same group demonstrated that pro-apoptotic *APAF1* and *CASP9* was not essential for anticancer drug sensitivity of *MYC*-induced lymphomas and did not promote oncogene-induced transformation of MEFs (Scott et al., 2004).

1.2.5 EBV

The Epstein–Barr virus (EBV) (named after British virologists Michael Anthony Epstein and Yvonne Murray Barr), also called human herpesvirus 4 (HHV-4), is a virus of the herpes family, which includes herpes simplex virus 1 and 2, and is one of the most common viruses in humans. It is best known as the cause of infectious mononucleosis, and a large part of the western world population is infected by the age of 20. It is also associated with particular forms of cancer, particularly Hodgkin's lymphoma, Burkitt lymphoma, nasopharyngeal carcinoma, and central nervous system lymphomas associated with human immunodeficiency virus (HIV). Furthermore, there is evidence that infection with the virus is associated with a higher risk of certain autoimmune diseases (James et al., 2001). Vaccines or specific therapy in treatment against EBV is currently not available.

The EBV penetrates cells, initially nasopharyngeal epithelial cells and B lymphocytes, by binding to the type 2 complement receptor CD21. At this stage, latent cycle genes produce a limited set of EBV proteins Epstein–Barr nuclear antigen (EBNA), latent membrane antigen (LMA), and terminal proteins (TPs). The virus reactivates occasionally, switching from latent to lytic cycle, with the production and expression of transactivating proteins, structural viral proteins, and envelope glycoproteins. EBV exerts a wide range of immune modulating effects, including inhibition of apoptosis, inhibition of the anti-EBV effects on interferon γ within B cells, and changes in the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1, and IL-6. In addition, EBV produces a viral IL-10 that allow the virus to limit the host immune response (Kanegane et al., 1997).

EBV is probably the most efficient transforming virus in culture and has been shown to be able to convert >50% of all target cells into continuously proliferating, latently infected lymphoblastoid cells (LCLs). Studies in LCLs revealed the expression of nine viral latent proteins and several non-coding RNAs that are under the transcriptional control of EBNA2 (Thorley-Lawson and Mann, 1985). Together, these cooperate to drive the newly infected resting cell to resemble an antigen-activated B lymphoblast. In combination with some of the latent genes (e.g., LMP1) EBNA2 constitutes the growth transcription program that contributes to the oncogenic actions of EBV (Thorley-Lawson and Gross, 2004).

1.2.6 Acute leukemia

The word leukemia originates from Greek; *leukos* meaning white, and *haima* meaning blood and describes an abnormal clonal expansion of hematological precursors in the bone marrow rendering a leakage of these immature cells to the peripheral blood. The two main groups of acute leukemia are acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) and afflicts both adults and children all over the world. Acute leukemia is somewhat more common in men and the incidence increases with age. At the same time acute leukemia and brain tumors account for 50 % of all childhood cancer in the western world (Gaynon et al., 2010).

Undoubtedly, the tools of molecular biology methods have increased the possibility of specifying the diagnosis for patients with acute leukemia. However, the foundation of all diagnostics in acute leukemia still relies of morphological examination of bone marrow smears. To further subgroup the acute leukemia the immunophenotype is determined by flow cytometry using a panel of antibodies. The antigens detected are markers for hematopoietic stem cells, B cells, T cells and myeloid cells etc. Cytogenetic evaluations contribute further to an adequate diagnosis and the most common translocations: t(4;11), t(6;11), t(9;11), t(11;19) and t(11;X) involves chromosome 11q23. These translocations all result in a number of oncogenic fusion proteins (Kagan, 1993).

Treatment outcome for children with acute myeloid leukemia (AML) and especially acute lymphoblastic leukemia (ALL) has improved substantially with the use of risk-directed treatment and improved supportive care. The 5-year event-free survival rates for ALL now range between 76% and 86% in children receiving protocol-based therapy in the developed countries (Gaynon et al., 2010, Moricke et al., 2010). By using cytostatic drugs in combination with drugs against the side effects (anemia, bleeding and infection) the aim is to completely cure the patients. The anticancer drugs includes anti-metabolites (e.g., methotrexate), anthracyclins (e.g., daunorubicin), vinca alkaloids (e.g., vincristine) (Lerner, 2011) etc.

1.3 CHEMOTHERAPY AND CHEMORESISTANCE

Anticancer drugs represent one out of three entities (i.e., drugs, surgery and irradiation) of treatment strategy for patients suffering from malignancies and include a broad range of agents. Conventional anticancer therapy relies on three cornerstones: 1) the knowledge of tumor responsiveness to the specific drug, 2) an understanding of the mechanism(s) underlying the cytotoxic effects as well as mechanisms of resistance, and 3) the pharmacokinetic features as well as side effects in normal tissue (Skipper et al., 1970). Undoubtedly, the main objective of anticancer treatment is to cure the patient and anticancer therapy has for a long time, at least in models of leukemia, been associated the "fraction cell kill hypothesis" (Berenbaum, 1972). This hypothesis is based on the fundamental assumption of constant fractional kill per treatment cycle. On the other hand, it is unlikely to be valid of slow growing heterogeneous solid tumors. Moreover, the growing but still somewhat controversial (associated to confusion regarding the definition) field of cancer stem cells (CSC) is emerging with implications for modern anticancer treatment. By using substances targeting the rare subpopulation of cells (i.e., CSC) by which the entire tumor is sustained complete eradication of the tumor might be achieved (Gupta et al., 2009). However, work with B lymphoma in nonirradiated histocompatible recipient mice from Strasser and co-workers suggest that tumor growth is not necessarily driven by CSC (Kelly et al., 2007).

Previous generations of today's anti-cancer drugs were brought to clinical trials already in the 1940's in the treatment of hematological malignancies. In using single agents such as nitrogen mustard, antifolates, corticosteroid and vinca alkaloids striking first responses were seen. Even though complete remission often was obtained relapse was common and always associated with resistance to therapy. Based on this the rationale combination therapy was introduced in treatment of human malignancies (Chabner, 2006).

For a favorable clinical outcome possible cross-resistance should be considered when formulating combination treatment therapies. Resistance to chemotherapy can be the result of inherent features of the tumor cells as well as the result of specific mutations. Examples of one unique mutation leading to cross-resistance are alterations in binding of inhibitors of enzymes that promote DNA strand breaks (e.g., topoisomerase II

inhibitors) in combinations of anthracyclins. In other respects, a single mutational change may lead to multidrug resistance as in the case of the *ABCB1* gene, also referred to as the MDR-1 gene, which encodes the P-170 membrane glycoprotein. This evolutionary conserved membrane protein pump promotes the efflux of natural toxins such as vinca alkaloids, anthracyclins, taxenes, actinomycin D and epipodophyllotoxins (Kartner et al., 1983). The *ABCB1* gene product belongs to a family of multi drug resistance proteins (MRPs) shown to be widely expressed in epithelial tumors (Ross and Doyle, 2004). Moreover, detoxification systems, including glutathione, have been demonstrated to be increased in tumor cells (Johansson et al., 2007).

1.3.1 Conventional chemotherapy

Virtually all chemotherapeutic drugs currently used induce apoptosis, when the apoptotic machinery of the cancer cells is intact (Fadeel and Orrenius, 2005). The first observation of apoptosis induction by antineoplastic drugs was reported more than 30 years ago in seminal work from Kerr and co-workers (Searle et al., 1975). The effects of most conventional antineoplastic drugs rely on the concept that tumor cells are rapidly dividing and that the DNA integrity is compromised. By using combination therapy targeting both the fraction of slowly dividing G_0 cells as well as the rapidly dividing cells in S phase, of for example solid tumor, effective response can be achieved. Furthermore, by the use of chemotherapy as neoadjuvant before surgical resection the bulk of tumors favorable clinical outcome can be accomplished. Some commonly used agents in anticancer treatment will be discussed below.

One of the best characterized and most versatile chemotherapeutic agents used treatment of both solid tumors and leukemia are antifolate compounds. The anticancer functions of folate metabolism inhibitors (e.g., methotrexate) are a consequence of the substitution of an amino group for the hydroxyl at position 4 of the pteridine ring and thereby inhibiting DNA synthesis. This will activate p53 dependent transcription, inducing apoptosis via extrinsic (Friesen et al., 1999) as well as intrinsic signaling (Savion et al., 2009). A common mechanism of intrinsic or acquired resistance of resistance to methotrexate is identified as defective uptake (i.e., plasma membrane transport) (Rodenhuis et al., 1986).

For more than 50 years guanine analogues (e.g., 6-mercaptopurine, 6-thioguanine and azathioprine) has been successfully used in treatment of childhood acute leukemia. More recently, cytidine analogues (e.g., arabinose nucleosides or Ara-C) has been incorporated in treatment regimens of AML, generally in combination with an anthracyclins. Ara-C induces tumor cell death by acting as an analogue of deoxycytidine, generating multiple effects on DNA synthesis, activating intrinsic apoptosis (Burnett et al., 2011). Resistance to Ara-C is coupled to mechanisms such as activity loss of deoxycytidine kinase, mutations in or over expressions of specific genes such as *WT1*, *CEBPA*, and the ratio of *BCL2* to *BAX* (Del Poeta et al., 2008) etc. Defects in apoptosome proteins, such as Apaf-1, are frequent in AML and treatment with 5-aza-2'-deoxycytidine, a specific inhibitor of DNA methylation, could restore

Apaf-1 expression and potentially increase efficiency of chemotherapeutic agents (Fu et al., 2003).

Another compound used in treatment of myeloproliferative disease (i.e., chronic myelogenous leukemia) is hydroxyurea (HU). Originally synthesized in the late 1800's it entered clinical trials in the 1960's (Stearns et al., 1963). Hydroxyurea exert its cytotoxic actions by targeting ribonucleotide reductase (RR) causing DNA damage and activate the ATM- and Rad3-related (ATR) kinase. DNA damage induces upregulation of p53 and recent studies suggest that mitogen-activated protein kinase (MAPK) family and play an important role (Wei et al., 2011).

Within the group of anthracyclin antibiotics daunorubicin and doxorubicin are among the most widely used anticancer agents in modern clinical practice. Discovered more than 40 years ago doxorubicin is primarily used in treatment of solid tumors including lymphoma (e.g., Hodgkin and non-Hodgkin lymphoma), while daunorubicin is used in treatment of acute leukemia (Dimarco et al., 1964). Anthracyclins show a high affinity for DNA, with a preference of dGdC-rich regions, and easily intercalates into the DNA helix (Chaires et al., 1990). The complete death inducing mechanisms of anthracyclins remain to be elucidated. However, doxorubicin was shown to result in caspase-dependent ceramide-mediated apoptosis (Jaffrezou et al., 1996, Gamen et al., 2000).

Originating from plant extracts used in traditional folk medicine, a number of semisynthetic derivatives with topoisomerase inhibitory effects have been produced. Under the brand name VePesid®, etoposide was approved for clinical use in 1984 (Sinkule, 1984). Etoposide is used as second-line regimens or salvage therapies in non-Hodgkin lymphomas including HIV-associated non-Hodgkin lymphomas (NHLs) (Sparano et al., 2004). The cytotoxic effect of topoisomerase II inhibitors is a result of stabilizing DNA cleavage complexes rather than preventing enzyme catalytic activity which will result in activation of the intrinsic apoptotic machinery. To this end, caspase-2 and Apaf-1 are suggested key players in etoposide-induced apoptosis (Lassus et al., 2002, Robertson et al., 2002, Sun et al., 2005).

1.3.2 Proteasome inhibitors

As opposed to conventional anticancer therapy the key target of novel anticancer drugs are functions and/or processes uniquely essential to the tumor cells. Novel drugs comprise structures such as macromolecules (e.g., monoclonal antibodies) to small molecule compounds. Examples of selective, tailored therapy targets presently in the clinic are the epidermal growth factor receptor (EGFR) (cetuximab), the epidermal growth factor receptor-tyrosine kinase (gefitinib), the *HER2/neu* human epidermal growth factor (trastuzimab), the *BCR/ABL* tyrosine kinase enzyme, angiogenesis (bevacizumab), and the proteasome (bortezomib, which will be discussed below). Promising future therapeutic targets lies within activating an inhibited apoptosis program of tumor cells, for example the Bcl-2/Bcl-XL inhibitor ABT-737 (Oltersdorf et al., 2005) and the activator of mutated p53 PRIMA-1 (Bykov et al., 2002).

The 26S proteasome is a large, multicatalytic protease that degrades polyubiquitinated proteins into small peptides (Figure 3). The main structure composes of two subcomplexes: a 20S core particle with catalytic activity and a regulatory 19S regulatory particle. Proteolysis of short-lived or regulatory proteins, from all compartments (i.e., cytoplasm, nucleus ER etc.) of the cell is predominantly executed by the proteasome. Conjugation of ubiquitin, a highly evolutionarily conserved 76-residue polypeptide, to the protein substrate is carried out via a three-step mechanism. Initially, the ubiquitin-activating enzyme E1 activates ubiquitin in an energy-dependent reaction. One of several E2 enzymes (ubiquitin-carrier proteins or ubiquitin-conjugating enzymes) transfers the activated ubiquitin from E1, to the substrate that is specifically bound to a member of the ubiquitin-protein ligase family, E3. There are a number of different classes of E3 enzymes. For example the RING finger-containing E3s (e.g., XIAP) catalyze direct transfer of the activated ubiquitin to the E3-bound substrate. By adding activated ubiquitin moieties to internal Lys residues on the previously conjugated ubiquitin molecule, a polyubiquitin chain is synthesized. This polyubiquitin chain can then be recognized by the 26S proteasome (Glickman and Ciechanover, 2002).

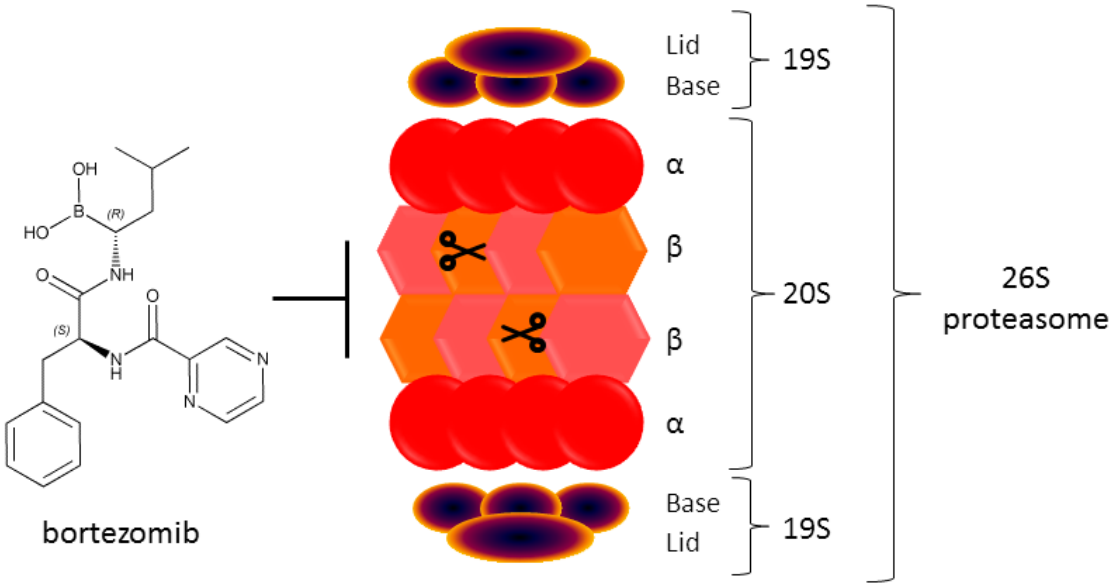

Figure 3. Inhibitory actions of proteasome inhibitor, bortezomib (Velcade®) on the human 26S proteasome.

Bortezomib (Velcade®), a dipeptidyl boronic acid, is a highly selective inhibitor of the 26S proteasome (Figure 3). It was approved in 2003 for treatment of relapsed refractory multiple myeloma (MM) and, more recently, for treatment of mantle cell lymphoma and newly diagnosed MM (Molineaux, 2011). It belongs to a second generation of proteasome inhibitors as opposed to Streptomyces metabolite lactacystin, a first generation proteasome inhibitor. Proteasome inhibitors constitute a reliable target for

cancer therapy due to the fact that the ubiquitin proteasome system is a highly conserved intracellular pathway. Moreover, the high abundance of proteasomes in certain types of cancers as well as in increased protein turnover in highly proliferating cells contributes to its efficacy (Kumatori et al., 1990). By inhibiting the proteasome these drugs affect essential cellular processes ranging from cell cycle regulation, proliferation to apoptosis. In the original publications by Anderson and colleagues on effects of bortezomib in multiple myeloma it was suggested that it induced cell death in NF-κB-dependent manner (Hideshima et al., 2001, Hideshima et al., 2002). However, NF-κB inhibition alone cannot explain the selective anti-cancer effect of bortezomib and has been demonstrated to act not only on cancer cells themselves, but also on stromal cells (Hideshima et al., 2001). Moreover, bortezomib has a chemosensitizing effect when administered together with other anti-cancer agents, an effect which may, in part, be due to the inhibition of NF-κB (Cusack et al., 2001, Russo et al., 2001, Bhalla et al., 2009). Recent studies have shown that bortezomib-induced apoptosis is dependent on factors involved in classical apoptotic signaling, such as the BH3-only protein Noxa (Smith et al., 2011). Although proteasome inhibitors comprise a promising group of anti-cancer drug reports of resistance are currently appearing in the literature. Mechanisms underlying bortezomib resistance are not fully understood but have been suggested to involve mutations of proteasomal subunits and Bcl-2 upregulation (Franke et al., 2011, Smith et al., 2011). Moreover, most of the studies to date were conducted on MM cells, but studies in leukemic cells are needed, as bortezomib is under consideration for treatment of such malignancies (Vink et al., 2006).

2 AIMS OF THE STUDY

The overall aim of the current thesis project is to elucidate mechanisms of resistance to apoptosis induction in B lymphoma and acute lymphoblastic leukemia. The specific aims are to determine:

- whether pharmacological and/or genetic manipulation of apoptotic protease-activating factor-1 (Apaf-1), second mitochondrial activator of caspases (Smac) and/or endogenous inhibitors of apoptosis (IAPs) can overcome chemoresistance in B lymphoma cells;

- the importance of Apaf-1 in response to proteasome inhibitor versus death receptor-mediated apoptosis in T cell leukemic cells;

- the expression of anti-apoptotic factor HS1-associated protein X1 (HAX-1) at the protein and transcript level in human B lymphomas;

- the responsiveness of chemoresistant B lymphoma cells to induction of NF-κB inhibition-induced apoptosis and the potential role of HAX-1 in apoptosis in B lymphoma cells.

3 MATERIALS AND METHODS

Detailed description of the techniques, materials and methods used in our studies can be found in the publications and manuscripts included in this thesis. The sections below provide a summary of the methods.

3.1 APOPTOSIS INDUCTION

For apoptosis induction we used staurosporine and lactacystin (Paper I) (Sigma-Aldrich, St Louis, MO), anti-Fas antibody clone CH11 (Paper II) (Medical and Biological Laboratories, Ltd, Nagoya, Japan), and TPCK and Bay-117082 (Paper IV) (Calbiochem, San Diego, CA). In addition etoposide (Bristol-Meyers, Stockholm, Sweden) was used as a control for the induction of intrinsic apoptosis (Paper IV).

3.2 CELL CULTURE

3.2.1 Cell lines

Raji, the first continuous human cell line of hematopoietic origin, was established in 1964 from a Nigerian Burkitt lymphoma patient by, and is positive for Epstein Barr virus (Pulvertaft, 1964). Raji cells lack a functional p53 gene as a result of point mutations on both alleles (Duthu et al., 1992) and are considered to be type I cells (Scaffidi et al., 1998). Cells were maintained in RPMI 1640 (Sigma) supplemented with 10% FBS, 1 mM L-glutamine and 100 U/mL penicillin/100 μg/mL streptomycin in a humidified incubator at 37^{o}C and 5% CO_2. Every third or fourth day the cells were counted, using tryphan blue 1:1 dilution, and resuspended in fresh medium to a density of 0,4 x 10^6 cells/ml, to keep them in a logarithmic growth phase (Paper I and IV).

The DG-75 cell line was established in 1977 by Eva Klein, George Klein and co-workers, and originated from a Burkitt lymphoma patient, negative for Epstein Barr virus (Ben-Bassat et al., 1977). Cells were maintained in RPMI 1640 supplemented with 10% FBS, 1 mM L-glutamine and 100 U/mL penicillin/100 μg/mL streptomycin in a humified incubator at 37^{o}C and 5% CO_2. Every third or fourth day the cells were counted, using tryphan blue 1:1 dilution, and resuspended in fresh medium to a density of 0,2-0,25 x 10^6 cells/ml, which keeps them in a logarithmic growth phase (Paper I and IV).

The type II cell line Jurkat originates from human acute T cell leukemia and was established in 1977 (Schneider et al., 1977). The Jurkat cell line is considered to have a non-functional p53 gene (Mansilla et al., 2006). Cells were maintained in RPMI 1640 supplemented with 10% FBS, 1 mM L-glutamine and 100 U/mL penicillin/100 μg/mL streptomycin in a humified incubator at 37^{o}C and 5% CO_2. Every third or fourth day the cells were counted, using tryphan blue 1:1 dilution, and resuspended in fresh medium to a density of 0,2-0,25 x 10^6 cells/ml, which keeps them in a logarithmic growth phase (Paper II).

3.2.2 Primary leukemic blasts

Primary leukemia blasts were obtained from children with acute leukemia at the Karolinska University Hospital. Informed consent was obtained from each parent in accordance with the conditions of the approval of the study by the local ethics committee (Stockholm, Sweden). Mononuclear cells were isolated from bone marrow samples by centrifugation on a Ficoll/Hypaque gradient (Lymphoprep, Oslo, Norway) and cells were cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide. Upon thawing, lymphoblasts were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technologies) as described previously (Laane et al., 2007) (Paper II).

3.3 APOPTOSIS DETECTION

3.3.1 Phosphatidyl serine exposure

Plasma membrane exposure of phosphatidyl serine (PS) was determined by flow cytometric detection of the PS-binding protein annexin V using the annexin V-FITC apoptosis detection kit (Oncogene Research Products, Cambridge, MA, USA). Briefly, cells were harvested, washed and resuspended in HEPES buffer (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl \cdot 6H_2O$, 1.8 mM $CaCl2 \cdot 6H_2O$) containing annexin V-FITC and propidium iodide (PI) before FACS analysis, as described previously (Karpova et al., 2006a) (Paper II).

3.3.2 Caspase-3 activation

Caspase-3 was fluorimetrically measured by cleavage of the fluorogenic substrate DEVD-AMC (Nicholson et al., 1995). Briefly, cells were harvested on ice and quickly frozen to -20^{o}C. Fifty micro molar DEVD-AMC (in DMSO) was dissolved in the reaction buffer (100 mM HEPES, 10% sucrose and 0.1% 3-[(3-Cholamidopropyl)+dimethylammonio]-1-propanesulfonate hydrate (CHAPS), pH 7,25) also containing 0.5% dithiothreitol (DTT), 0.01% Nonidet® P 40 (NP-40), and added to the cells. Product of the enzymatic reactions was measured in real time of enzyme-catalyzed release of AMC using Tecan Infinite F200 plate reader (Tecan Group Ltd, Männedorf, Switzerland) operating with Magellan V6.5 software (Tecan Group Ltd, Männedorf, Switzerland) (Paper I, II and IV).

3.3.3 Nuclear morphology changes

Nuclear apoptosis was assessed as described previously (Karpova et al., 2006b). Briefly, cells were harvested and fixed in 2% paraformaldehyde. Cytospin preparations were stained with 1µg/mL Hoechst 33342. Apoptotic features (fragmented/condensed nuclei) of the cells were scored using fluorescence microscope (Paper I and IV).

3.3.4 Hypodiploid DNA content

Propidium iodide (PI) can be used to quantify the cellular DNA content and cellular fluorescence can then be measured using flow cytometry (Nicoletti et al., 1991). Briefly, cells were harvested, washed in PBS, pelleted and resuspended in 0.5 mL PBS

based staining solution containing 50 μg/mL PI, 0.1% Triton X-100, and 0.1% sodium citrate, and analyzed by FACS (Paper I, II and IV).

3.3.5 Mitochondrial membrane potential

Loss of mitochondrial membrane potential (MMP) was detected using tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) (Jitkaew et al., 2009). Briefly, 20 min prior to harvesting the cells, 1 μL (TMRE 25 nM) was added to the cell cultures. After washing the cells with PBS, HEPES buffer (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM MgCl · $6H_2O$, 1.8 mM CaCl2 · $6H_2O$) containing TMRE was added and the samples were analyzed by FACS (Paper II and IV).

3.4 OTHER *IN VITRO* METHODS

3.4.1 Gel electrophoresis and western blot

For total cell lysates, cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10 % glycerol, 1 % NP-40, and 0.5 % deoxycholate). Protein concentration was measured using the BCA™ Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL, USA). For cellular fractionation, 10^6 cells were incubated on 5 min in KCl buffer (150 mM KCl, 1 mM MgCl in 5 mM Tris pH 7.4) supplemented with 0.01 % digitonin, centrifuged, and supernatant (cytosolic fraction) and pellet (enriched mitochondrial fraction) was collected. Thirty microgram protein of each sample was loaded onto a sodium dodecyl sulfate (SDS)-gel, transferred onto polyvinylidene diflouride membranes and probed with the indicated antibodies. Proteins were visualized by enhanced chemiluminescence (ECL). The films were scanned, and densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (Papers I, II and IV).

3.4.2 DNA damage assessment

The Comet assay is utilized to monitor DNA damage (Kobayashi H, 1995). In brief, cells were harvested, washed with PBS, applied in 0.75 % Low Melting Point Agarose (LMPA) to pre-coated agarose glass slides, and lysed in lysis buffer (10 mM Tris, 2.5 M NaCl, 100 mM EDTA). Glass slides were calibrated in electrophoresis buffer (10 N NaCl, 200 mM EDTA), run at 20 V and 150 mA for 20 min, neutralized with Neutralization Buffer (0.4 M Tris-HCl), stained with ethidium bromide and mounted on glass slides. At least 100 cells were counted for each sample at x10 or x40 magnification using an inverted fluorescence microscope, and the % of cells with comet tails were determined (Paper II).

3.4.3 Reactive oxygen species (ROS) measurements

Production of superoxide was measured using dihydroethidium (DHE, Molecular Probes, Leiden, The Netherlands) as described elsewhere (Jitkaew et al., 2009). Oxidation of DHE by superoxide anion yields the fluorescent ethidium. Briefly, cells were incubated with 5 μM DHE in RPMI 1640 medium for 30 min at 37ºC. After resuspension in PBS, cells were submitted to FACS analysis using FACScan (Becton Dickinson) (Paper IV).

3.4.4 Immunofluorescent labeling of cells

Localization of proteins was determined using immunocytochemistry and fluorescence microscopy. In brief, the cells were treated, fixed in 4% formaldehyde, permeabilized and blocked in 2% bovine serum albumin in 0.05% Triton X-100. The cells were incubated over night with primary antibody and with fluorescent probe-conjugated secondary antibody (Molecular Probes, Leiden, The Netherlands) for 1 h at room temperature, then stained with mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA). The protein localization was evaluated using a Nikon ECLIPSE TE-2000-S fluorescence microscope (Nikon Corp.) equipped with a DS-5M digital camera operating with NIS-Elements software (Nikon Instruments, Badhoeverdorp, The Netherlands) (Paper IV).

3.5 GENE EXPRESSION MICROARRAY ANALYSES AND BIOINFORMATICS

Global gene expression profiling is a powerful strategy for improved understanding of the underlying pathogenesis and biology of human cancer (Quackenbush, 2006). Recent assembly of microarray data into databases provides means to assess the expression of single genes or groups of genes across multiple tissues and disease states. In paper II we utilized the Human Gene Expression Map, an online resource constructed from the integration of 5372 gene expression analyses of 369 different cell and tissue types, disease states and cell lines (Lukk et al., 2010). Statistical analyses of the three probes targeting the *APAF1* gene on the Affymetrix HG-U133A were performed using one-way ANOVA with multiple group-wise comparisons to the global mean, and post-hoc tests were applied to identify selected comparisons and globally adjusted p-values. In paper III the In Silico Transcriptomics (IST) database containing data from 9,783 Affymetrix gene expression analyses of 43 normal tissues, 68 cancer types and 64 other diseases was used (Kilpinen et al., 2008).

In paper IV total RNA was extracted from three separate experiments of unexposed and compound-exposed cells, respectively using Trizol reagent (Invitrogen, Carlsbad, CA) and the Qiagen RNeasy Mini columns (Qiagen, Chatsworth, CA) according to the manufacturers' instructions. RNA concentration were determined using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and RNA quality was determined using the RNA 6000 Nano LabChip kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Purified RNA (RIN value $\geq$7) was amplified with the Illumina RNA TotalPrep Amplification kit (Ambion, Austin, TX) and the labeled cRNA was hybridized to HumanHT-12 v3 Expression BeadChips (Illumina Inc, San Diego, CA). Arrays were scanned with the Illumina BeadArray reader. The raw gene expression data were quantile-normalized and analyzed using the BioArray Software Environment (BASE) software (Vallon-Christersson et al., 2009) and R (www.r-project.org). The Illumina probes were re-annotated using Re-annotation and Mapping for Oligonucleotide Array Technologies (ReMOAT) (Barbosa-Morais et al., 2010), and for the ontology studies only probes with good or perfect quality were used.

The differentially expressed transcripts from the compound-exposed cells relative to the un-exposed state were further mined by Gene Ontology (GO) categorization and

network analysis. The web-based functional annotation tool kit DAVID Bioinformatics Resources 6.7 (Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov/) was utilized to sort the transcripts based on GO categories. For each GO term the probability values were estimated based on the hypergeometric distribution by comparing the number of differentially expressed genes with the expected number of such genes as reference. The gene lists including significant differentially expressed genes were compared to a reference background containing genes representing probes with good or perfect quality according to Barbosa-Morais et al (Barbosa-Morais et al., 2010). Enriched categories encompassing at least 5% of the differentially expressed genes were selected from the analyses.

Ingenuity Pathway Analysis (IPA) was applied to sort the differentially expressed genes into biological networks (http://www.ingenuity.com). Molecular networks for direct relationships were generated from data in the IPA knowledge base by uploading selected transcripts termed "focus genes" and corresponding expression values ("mean fold change"). Fisher's exact test was applied for ranking and significance analysis of the focus genes in the network and a score of three ($p<0.001$) was set as a threshold, including association of the data to the most significant biological functions (termed "top functions").

4 RESULTS

4.1 PAPER I: SMAC-MEDIATED SENSITIZATION OF HUMAN B-LYMPHOMA CELLS TO STAUROSPORINE- AND LACTACYSTIN-TRIGGERED APOPTOSIS IS APOPTOSOME-DEPENDENT

In this study the requirement of Apaf-1 for Smac-dependent potentiation of staurosporine- and lactacystin-triggered apoptosis was investigated. In addition, sensitization to Apaf-1-dependent caspase activation upon immunodepletion of inhibitor of caspase, cIAP2 was studied. Initially, we confirmed that Raji and DG-75 display complete resistance to staurosporine (STS) and lactacystin compared to a panel of Burkitt lymphoma (BL) cells. IAPs are highly expressed in several types of human cancer and studies in Hodgkin lymphoma B cell lines have demonstrated high levels of XIAP as a mechanism of cytochrome *c*-dependent apoptosis resistance (Kashkar et al., 2003, Wright and Duckett, 2005). We thus examined the protein expression of cIAP1, cIAP2 and XIAP in a panel of BL cells as well as apoptosis sensitive T-cell leukemic cell line Jurkat, and found that the expression of cIAP2 was elevated compared to Jurkat cells. To determine whether cIAP2 plays any role in the resistance to apoptosis in BL cells, cellular extracts from Raji cells immunodepleted for cIAP2 were examined for cytochrome *c*-dependent caspase activation, but remained refractory.

Our group has previously shown that cytosolic expression of Apaf-1 plays a key role in cytostatic drug-induced apoptosis in BL cells (Sun et al., 2005). To further elucidate the mechanisms of apoptosis resistance in BL cells, cellular extracts from Raji cells overexpressing cytosolic Apaf-1 or mock transfected were immunodepleted for cIAP2. When examined for cytochrome *c*-dependent caspase activation we found immunodepletion of cIAP2 to potentiate caspase activation in Raji cells stably transfected with cytosolic Apaf-1.

In a previous study from our group (Sun et al., 2005) as well as in this study we could conclude that the apoptosis resistant BL cells were able to release cytochrome *c* from mitochondria to the same extent as apoptosis sensitive Jurkat cells. Accordingly, the resistance to apoptosis induction in these cells involved other factors. Thus, we examined the release of pro-apoptotic factor Smac from mitochondria after treatment with STS and found the mitochondrial fraction of Smac to remain constant over time in Raji cells as opposed to Jurkat cells. To further elucidate the importance of Smac in the defective apoptosis signaling in Raji cells synthetic Smac-peptides were introduced to our system of cells overexpressing cytosolic Apaf-1 or not. Two different types of peptides were introduced; synthetic Smac-peptides conjugated with fluorescent probe FITC and FITC-conjugated control peptide expressing a scrambled amino acid sequence, or a non-fluorescently labeled synthetic Smac-peptide. Both types of Smac-peptides were able to potentiate apoptosis in Raji cells overexpressing cytosolic Apaf-1 in response to STS and lactacystin.

The importance of Apaf-1 in apoptosis signaling was further supported as the lipid-raft disrupting agent methyl-β-cyclodextrin, releasing Apaf-1 from its mislocalization in lipid rafts in the plasma membrane, could sensitize DG-75 cells to STS- and lactacystin-induced apoptosis.

In summary we have provided evidence that cytosolic Apaf-1 expression is required for Smac-dependent potentiation of apoptosis of BL cells. Endogenous caspase inhibitor cIAP2 was demonstrated to serve as a modulator of sensitivity towards cytochrome c-dependent caspase activation.

4.2 PAPER II: REQUIREMENT OF APOPTOTIC PROTEASE-ACTIVATING FACTOR-1 FOR BORTEZOMIB-INDUCED APOPTOSIS BUT NOT FOR FAS-MEDIATED APOPTOSIS IN HUMAN LEUKEMIC CELLS

In paper I (Sun et al., 2007) we studied the importance of Apaf-1 in microbial derived proteasome inhibitor lactacystin-induced apoptosis in BL cell lines. The proteasome inhibitor bortezomib has been approved for treatment of relapsed refractory multiple myeloma and, more recently, for treatment of mantle cell lymphoma. Clinical trials are underway to assess the efficacy of bortezomib in several other human malignancies including leukemia (Vink et al., 2006). Human leukemic cells express abnormally high levels of proteasomes compared with normal peripheral blood cells (Kumatori et al., 1990) and leukemic cells are significantly more sensitive to proteasome inhibition than normal bone marrow progenitor cells or peripheral blood lymphocytes (Masdehors et al., 1999, Soligo et al., 2001). For this reason, we aimed and elucidated the importance of Apaf-1 for bortezomib-induced apoptosis using the T-cell leukemic Jurkat cell line. Treatment with bortezomib induced induction of the pro-apoptotic factor Noxa upstream of mitochondria, drop in MMP and release of cytochrome *c* from mitochondria in cells with or without Apaf-1. However, we only observed caspase-3-like activity, PS-exposure, cells with hypodiploid DNA and PARP cleavage in cells with shRNA against Apaf-1 expression.

For comparison, Jurkat cells with or without Apaf-1 expression were treated with agonistic anti-Fas antibodies to activate classical, death receptor-mediated apoptosis. Jurkat cells stably transfected with shRNA against Apaf-1 or with shRNA with mock sequence treated with agonistic anti-Fas antibody both induced apoptosis. Consequently, Fas-induced apoptosis in Jurkat (type II) appeared to be Apaf-1-independent (and hence, cytochrome *c*-independent). However, cell death may still very well depend on the release of mitochondrial factors, such as Smac and/or omi/HtrA2, as suggested previously (Shawgo et al., 2009).

Interestingly, Jia et al. have previously reported that Apaf-1 deficiency is one mechanism underlying primary ALL blast resistance to cytochrome c-induced activation of caspase-3 (Jia et al., 2003b). For this reason, we examined Apaf-1 protein expression in leukemic blasts from pediatric patients with ALL, and assessed sensitivity

towards bortezomib-induced apoptosis. We found that the expression of Apaf-1 varied in a small panel of leukemic blasts from patients with ALL. Although sample number was low and assessing correlation of the responsiveness to bortezomib with Apaf-1 expression difficult, we noted the lowest increase in apoptosis in response to bortezomib in the patient sample completely deficient for Apaf-1.

Finally, in light of the fact that Apaf-1 expression appeared to be a key determinant of bortezomib-induced apoptosis in the Jurkat model, we explored the Human Gene Expression Map for the expression of Apaf-1 transcripts in a diverse range of human malignancies (Lukk et al., 2010). Assessment of the six leukemic subtypes relative to 96 biological groups demonstrated consistently increased expression of Apaf-1 in acute lymphoblastic leukemia, acute myeloid leukemia, chronic myeloid leukemia, and precursor T lymphoblastic leukemia. Assessment of the 18 cell lines in the blood neoplasm cell line group, which also encompasses the Jurkat cell line, indicated similar Apaf-1 expression levels, with exception for the promyelocytic leukemic cell line HL60 that showed increased levels of Apaf-1.

Taken together, these studies showed that Apaf-1 were required for the pro-apoptotic effects of bortezomib in the Jurkat T cell leukemic cell line. Our results also showed that bortezomib induced apoptosis by regulating pathways that are mechanistically different from those activated upon death receptor ligation. Furthermore, *in silico* analyses of public transcriptomics databases indicated elevated Apaf-1 expression in several hematological malignancies, including acute lymphoblastic and myeloid leukemia. We also noted variable Apaf-1 expression in a panel of samples from patients with acute lymphoblastic leukemia. Our results suggest that the expression of Apaf-1 may be predictive of the response to proteasome inhibition.

4.3 PAPER III: HAX-1 EXPRESSION IN HUMAN B LYMPHOMA: ASSOCIATION WITH PROLIFERATION AND INVERSE CORRELATION WITH BCL-2 EXPRESSION

In the third paper, we aimed to study the expression of HAX-1 in human B lymphoma on transcript and protein level. HAX-1 is a multifunctional protein with ubiquitous expression and evident roles in apoptosis and cell migration (Fadeel and Grzybowska, 2009). However, HAX-1 expression has only been studied in a limited number of different cancer types and never in hematological malignancies. For this reason we determined the expression of *HAX1* in two public transcriptomics database, the In Silico transcriptomics (IST) database, containing data from 9,783 Affymetrix gene expression analyses of 43 normal tissues, 68 cancer types and 64 other diseases, and the Human gene expression map derived from 5,372 samples of 369 different cell and tissue types including various disease states (Lukk et al., 2010, Kilpinen et al., 2008). In particular B-cell-related hematopoietic neoplasms malignancies, such as plasma-cell leukemia and B-cell lymphomas, showed a high expression of *HAX1* using the IST data base. *HAX1* was significantly overexpressed in hematological malignancies such as anaplastic large cell lymphoma when interrogating the Human gene expression map.

The Bcl-2 proto-oncogene was first described as a molecular feature of follicular B cell lymphoma (Yang and Korsmeyer, 1996), and for comparison we also examined the expression of *BCL2* in the two public transcriptomics databases.

To elucidate the protein expression of HAX-1 in B-cell derived hematological neoplasms a panel of 50 B lymphomas, including B-CLL, Immunocytoma, MALT lymphoma, Mantle cell lymphoma, Follicular lymphoma, DLBCL, Burkitt lymphoma and Hodgkin lymphoma was screened for expression of HAX-1. HAX-1 was shown to be highly expressed in DLBCL and Burkitt lymphoma both representing a lymphoma type with a high proliferative index measured by expression of Ki-67. Both types of lymphoma also displayed low levels of Bcl-2. The follicular lymphoma samples were selected upon mRNA expression of *HAX1* retrieved from a previous publication by our group on microarray analysis on a cohort of follicular lymphoma (Björck et al., 2005). Three samples with high mRNA expression of *HAX1* and three samples with low expression of *HAX1* were selected from the cohort for the immunohistochemical staining. In follicular lymphomas that normally overexpress Bcl-2, we observed one samples with high expression of HAX-1 that were negative for Bcl-2. We then assessed the relative transcript levels of *HAX1* and *BCL2* using the complete dataset from a previous publication (Björck et al., 2005), and were able to demonstrate a negative correlation between *HAX1* and *BCL2* expression at the mRNA level.

To conclude, our data showed varying expression of HAX-1 on protein and transcript level in different sub-groups of human B lymphoma. Elevated levels of HAX-1 could also be correlated to a high proliferative index at protein level and to low expression of Bcl-2 on protein and transcript level in follicular lymphoma.

4.4 PAPER IV: N^{α}-TOSYL-L-PHENYLALANINE CHLOROMETHYLKETONE INDUCES CASPASE-INDEPENDENT APOPTOSIS IN CHEMORESISTANT BURKITT LYMPHOMA CELLS

These studies were aimed at elucidating the mechanisms of chemoresistance in BL further using the specific inhibitor of chymotrypsin-like serine proteases, N^{α}-tosyl-L-phenylalanine chloromethylketone (TPCK) and the NF-κB inhibitor Bay-11 7082 (Bay-11). The apoptosis-inducing ability by inhibition of NF-κB has been demonstrated in a variety of BL cell lines in the past decade (Zou et al., 2007, Klapproth et al., 2009, Nazari et al., 2011). Moreover, in a previous study by our group we reported that TPCK induces NF-κB-mediated caspase-dependent apoptosis in normal EBV-transformed B-cells (Jitkaew et al., 2009). Apoptosis signal transduction downstream of mitochondria in the cell lines used in this study, Raji and DG-75 was previously shown to be impaired in response to etoposide (Sun et al., 2005). In contrast, when treating Raji and DG-75 cells with TPCK and Bay-11 we found that both compounds induced dose- and time dependent apoptosis in both cell lines. The apoptosis, measured by drop in MMP and percentage cells with hypodiploid DNA could not be blocked using the pan-caspase inhibitor zVAD-fmk and no activation of caspase-3 was seen.

Based on the findings that BL cells expresses constitutive NF-κB binding activity in the nucleus, and have been suggested to be sensitive to NF-κB inhibition-induced cell death (Zou et al., 2007, Klapproth et al., 2009, Nazari et al., 2011) we hypothesized that the two compounds exerted their apoptotic actions in a similar fashion. For this reason we explored the effects on nuclear NF-κB expression after treatment with TPCK and Bay-11. Both treatments caused a decrease in the nuclear expression of NF-κB in Raji cells. Subsequently the cells were treated with TPCK or Bay-11 and the expression of NF-κB was assessed using fluorescence microscope evaluation. After 6 h the control cells in both cell lines displayed a clear nuclear localization. In contrast both treatments induced substantial cytosolic localization of NF-κB after both treatments.

We previously published data demonstrating the decrease in the expression of HAX-1 on protein and transcript level as a result of transcriptional regulation by NF-κB (Jitkaew et al., 2009). For this reason we examined the protein expression of HAX-1 in Raji and DG-75 cells after treatment with increasing doses of TPCK and Bay-11. Both treatments reduced the protein expression of HAX-1 in a dose-dependent fashion. However, at the highest doses of TPCK and Bay-11 not only the expression of HAX-1 but also β-actin was reduced. Moreover, we assessed the effect of TPCK and Bay-117082 on the expression of *HAX1* splice variant I and II (Carlsson et al., 2008) using quantitative real-time PCR. The expression of splice variant II was reduced after 1 h while the expression of splice variant I was unaffected.

In conclusion, we demonstrate the caspase-independent apoptosis-inducing effects of TPCK and Bay-11 on chemoresistant BL cells Raji and DG-75. Our data suggest that both compounds induce a decrease in constitutive NF-κB activity as well as reducing protein and mRNA expression of NF-κB target HAX-1, which may contribute to the sensitization to apoptosis. However, other effects of TPCK cannot be excluded. Future studies aimed at targeting silencing of HAX-1 expression may be informative. For comparison, B cells from patients with homozygos *HAX1* mutations are more sensitive to TPCK induced apoptosis (Jitkaew et al., 2009).

5 DISCUSSION

Apoptosis resistance contributes to carcinogenesis and chemoresistance, and acquired resistance towards apoptosis is a hallmark of most if not all types of cancer (Hanahan and Weinberg, 2011).

One of the key players in mitochondria-mediated apoptosis, Apaf-1, has been shown to be involved in mechanisms of resistance to chemotherapeutic drugs in B lymphoma and leukemia models (Sun et al., 2005, Franklin and Robertson, 2007). A reduced cytosolic expression of Apaf-1 was the result of a mislocalization of Apaf-1 to lipid rafts in the plasma membrane and the mechanism of resistance in B lymphoma cells (Sun et al., 2005). The same mechanism could be applied for the resistance to protein kinase C inhibitor staurosporine (STS) - and proteasome inhibitor lactacystin-induced apoptosis in Burkitt lymphoma cells as well (Paper I). The effect of staurosporine and lactacystin could be significantly increased by the use of synthetic Smac peptides that in turn blocked the effect of IAPs in these cells. Furthermore, depletion of cIAP2 with monoclonal antibodies sensitized these cells to caspase activation in an Apaf-1-dependent manner. Members of the IAP family are abundantly expressed in human tumors and one member, Survivin, has been commonly found in human tumor tissue but not in normal, terminally differentiated adult tissue (Deveraux and Reed, 1999). Moreover, interrogating the IST data base (Kilpinen et al., 2008) the relative mRNA expression of *BIRC4* (XIAP) is increased primarily in hematological malignancies such as acute and chronic leukemia and myeloma, further supporting the potential for targeting IAPs in anti-cancer treatment. Possible prospects of synthetic Smac peptides in cancer treatment in combination with activators of extrinsic and intrinsic apoptosis signaling have been reported by other groups in xenograft models of malignant glioma (Fulda et al., 2002) and non-small cell lung cancer (Yang et al., 2003). The requirement of an intact apoptosome-signaling pathway in Smac-mediated apoptosis has been described in acute myeloid leukemia (AML) (Jia et al., 2003a). Upon treatment with synthetic Smac peptides proliferation was inhibited and the cells displayed cell cycle arrest, indicating that Smac peptides potentially could affect rapidly proliferating cells as well as indolent stem-like tumor cells. However, sufficient protein expression levels of Apaf-1 were required for efficient killing of the cells. An intact Apaf-1 protein expression in its adequate cellular compartment thus seems to be essential in apoptosis induction by various extrinsic and intrinsic stimuli. Hypermethylation of the Apaf-1 promoter has been demonstrated in AML and ALL as well as several other different types of cancer (Fadeel et al., 2008). ALL samples completely deficient for Apaf-1 were seen also in our study (Paper II); however, the mechanism for this was not examined. Consequently, upregulation of Apaf-1 protein expression by demethylation treatment might provide a potential strategy to sensitize leukemic cells to chemotherapy-induced apoptosis. Apaf-1 is a key player in responsiveness to apoptosis by not only classical chemotherapeutic agents but also to novel anticancer drugs such as proteasome inhibitors (Almond et al., 2001, Nikrad et al., 2005, Smith et al., 2011). The proteasome inhibitor bortezomib (Velcade®) is currently approved by the US Food

and Drug Administration (FDA) in treatment of relapsed refractory multiple myeloma and mantle cell myeloma. The rationale for extending the use of bortezomib to other hematological malignancies such as acute leukemia is not farfetched since the levels of proteasomes in human ALL and AML cells as well as CML cells is much greater than in normal peripheral blood cells (Vink et al., 2006). The combined information from our research as well as the research of other groups stipulates Apaf-1 as a determinant for responsiveness to apoptosis induced by cytostatic drugs as well as novel anticancer regimes in hematological malignancies.

When classical caspase-dependent apoptosis signaling is perturbed cells may still succumb to apoptotic cell death. Caspase-independent mechanisms of cell death have been extensively described and involve different factors depending on the apoptosis inducing compound and cell type. Although the Bcl-2 homology of HAX-1 has been debated reports on the anti-apoptotic features of HAX-1 are manifold (Fadeel and Grzybowska, 2009). We suggest a potential redundancy of the two proteins as elevated levels of HAX-1 is correlated to a low expression of Bcl-2 in human B lymphoma (Paper III). The positive correlation with proliferation marker Ki67 might reflect the multi-functionality of HAX-1 and its suggested involvement in cancer cell migration (Ramsay et al., 2007). Additional mechanistic studies are needed in order to elucidate the role(s) of HAX-1 in the fate of tumor cells. However, determination of HAX-1 expression on transcript and protein level might be of clinical significance in diagnosis and outcome of certain types of B lymphoma. Previous studies by members of our research group have indicated the role of HAX-1 in serine protease inhibitor, TPCK-induced caspase-dependent cell death of normal EBV-transformed B cells (Jitkaew et al., 2009). Moreover, the expression of HAX-1 was suggested to be under transcriptional control of NF-κB. Indeed, this was supported in our study (Paper IV) as decreased nuclear activity of NF-κB was associated with a reduction in the expression of HAX-1 on protein and transcript level and coincided with induction of apoptosis of chemoresistant B lymphoma cell lines. Apoptosis induction by TPCK was caspase-independent and did not involve cleavage of known substrates in caspase-dependent apoptosis signaling. This suggests that HAX-1 could be involved in apoptosis induction involving several different mechanisms with and without the involvement of caspases. The diversity of the functional attributes as well as diversity in cellular localization of HAX1 can most probably be explained by alternative splicing of the *HAX1* gene. In a study by Trebinska and co-workers the expression of splice variant I and II was examined in panel of samples from patients with breast cancer. Notably, the variant I/variant II ratio was higher in normal tissue than in tumor tissue, and the relative overexpression ratio (tumor/normal) showed a higher overexpression of variant II in tumors than in normal tissue (Trebinska et al., 2010). This indicates that the expression of the different splice variants of HAX1 might exert different actions in normal versus tumor tissue. However, our findings do not exclude other possible perturbing characteristics of TPCK important in circumventing chemoresistance in Burkitt lymphoma cells. Altered redox status (i.e., oxidation) of cellular thiols has previously been demonstrated to trigger Bcl-2-independent drop in mitochondrial membrane potential and subsequent intrinsic apoptosis (Costantini et al., 2000). Due to the cellular localization of HAX-1 to membranes such as the ER-membrane and the nuclear membrane, but primarily to the mitochondrial membrane one might speculate that the

latter localization is imperative for the anti-apoptotic functions of HAX-1. Moreover, some studies suggest that Burkitt lymphoma cells display limited uptake capacity for cystine, and are thus prone to oxidative stress-induced cell death (Banjac et al., 2008). TPCK-induced apoptosis in our study was associated with the production of reactive oxygen species (ROS). Nevertheless, the cell death could be inhibited by co-treatment with not only glutathione precursor N-acetyl-cystein but also by L- and D-cystein, suggesting direct post-translational modifications rather than ROS production as a mechanism for apoptosis.

The studies in the present thesis are aimed at elucidating mechanisms of resistance to apoptosis induced by anticancer agents in B lymphoma and acute leukemia. For this purpose we have primarily used models of highly aggressive haematological malignancies commonly occurring in children; Burkitt lymphoma and acute lymphoblastic leukemia. The overall survival of patients suffering from these neoplasms has increased remarkably during the past three decades with the introduction of efficient treatment strategies (Magrath et al., 1996, Gaynon et al., 2010). These strategies include cycles of intensive therapy targeting rapidly diving cells, with limitless proliferative potential and decreased DNA integrity, affecting the bulk of the tumor cells. Slowly dividing or non-cycling tumor stem-like cells will not be affected and can possibly give rise to relapsed disease. Indolent lymphomas, such as follicular lymphoma, have demonstrated to be defective in their apoptotic signaling rather than in their inability to stop proliferating. In this context novel treatment strategies are needed for patients with inherent resistance to cytostatic drugs as well as for patients with chemoresistance-association relapsed disease. In this thesis we have utilized a combination of cell lines and primary patient-derived cells. Moreover, our studies on different factors involved in apoptosis resistance have been conducted using *in vitro* methods as well as *in silico* approaches. By focusing our research on reactivating the apoptotic program or inhibiting the anti-apoptotic machinery of tumor cells, such tailored treatment regimens might be achieved in the future.

6 CONCLUSIONS

In summary, the main results obtained from this thesis suggest the following:

- Apaf-1 is a determinant of responsiveness to conventional chemotherapeutic drugs, indicating possible biomarker function for clinical outcome in hematological malignancies

- Apaf-1 is a determinant of responsiveness to novel anticancer drugs i.e., proteasome inhibitors in hematological malignancies

- HAX-1 expression is elevated in B lymphomas on transcript and protein level and show negative correlation to Bcl-2 expression and positive correlation to Ki67 expression

- Caspase-independent apoptosis, in association with NF-κB inhibition and downregulation of HAX-1, provide means of circumventing chemoresistance in B lymphoma cells

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